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HIV SUSCEPTIBILITY FACTORS IN THE HUMAN GENITAL MUCOSA

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To my family with love

ABSTRACT

Heterosexual HIV transmission is the most common viral transmission route worldwide. To establish a persistent infection the virus needs to cross the mucosal surface of the genital tract. The genital mucosa is thus considered to be the portal of HIV entry and initial site of viral replication. A better understanding of the immunological milieu at the portal of viral entry is crucial for the development of preventive interventions.

In **Paper I** we investigated how herpes simplex virus 2 (HSV-2) affects the genital epithelial barrier and mucosal immune response in an HSV-2 seropositive, asymptomatic vs. HSV-2 seronegative male population in Kenya. The two study groups had comparable levels of all selected markers of inflammation and epithelial integrity, except for lower mRNA levels of the epithelial junction protein claudin-1 in the HSV-2 seropositive group, which may indicate a less robust genital epithelial barrier.

In **Paper II**, we investigated how the use of progesterone-based hormonal contraceptives affects the genital epithelial barrier and mucosal HIV receptor expression in healthy Swedish women. The progesterone-based intrauterine device (pIUD) group was compared to a non-hormonal contraceptive (noHC) group and a combined oral hormonal contraceptives (COC) group. Similar protein expression levels of HIV receptors and co-receptors were observed in the three study groups. However, women using pIUD displayed a thinner apical layer of the ectocervical epithelium and lower mRNA levels of the epithelial junction protein ZO-1 as compared to the control groups. These results suggest that pIUD use may weaken the ectocervical epithelial barrier against invading pathogens, such as HIV.

In **Paper III**, we further investigated how the use of hormonal contraceptives affects the production of antimicrobial peptides (AMPs) in different compartments of the female genital mucosa, including secretions and tissue. Women using COC had significantly lower mRNA levels of the AMPs BD-2 and trappin-2 in ectocervical tissue as compared to pIUD users. The two groups showed no differences in AMP protein expression in neither cervicovaginal secretion (CVS) nor in ectocervical tissue. These results suggest that the impact of sex hormones on local immune defences varies in tissue vs. secretions in the female genital tract.

In **Paper IV** we examined if epithelial thickness and/or the quantity and localization of HIV target cells in ectocervical epithelium is associated to the relative resistance of HIV exposed seronegative (HESN) women. Thus, female sex workers defined as HESN were compared to control women who were relatively new to sex-work. Our results show that the HESN phenotype is not associated with an altered epithelial thickness or with altered levels or distributions of HIV target cells in the ectocervical epithelium.

In summary, the studies characterized epithelial integrity in both the male and female genital tract, as well as the localization, distribution and quantity of immune cells and proteins in both tissue and secretions. These factors may be of importance for HIV susceptibility and was compared between study groups, characterized by various risk factors for HIV infection including HSV-2 infection, different types of hormonal contraceptive use and a phenotype of relative HIV resistance. Our results imply that the genital mucosa is a complex site and it is therefore of major importance to study the local immunological milieu in the tissues and not solely in secretions, which opens up for a much more comprehensive picture. Further, our data indicates that strengthening the genital epithelial barrier of the local genital mucosa may be a beneficial way to reduce sexual transmission of HIV, and should thus be incorporated in potential future prevention strategies against HIV infection.

LIST OF SCIENTIFIC PAPERS

- I. **Comparable mRNA expression of inflammatory markers but lower claudin-1 mRNA levels in foreskin tissue of HSV-2 seropositive versus seronegative asymptomatic Kenyan young men**
MARIA RÖHL, Annelie Tjernlund, Supriya D. Mehta, Pernilla Petersson, Robert C. Bailey and Kristina Broliden
BMJ Open. 2015 Feb 18; 5(2):e006627
- II. **Progesterone-Based Intrauterine Device Use Is Associated with a Thinner Apical Layer of the Human Ectocervical Epithelium and a Lower ZO-1 mRNA Expression**
Annelie Tjernlund, Ann Marie Carias, Sonia Andersson, Susanna Gustafsson-Sanchez, MARIA RÖHL, Pernilla Petersson, Andrea Introini, Thomas J. Hope and Kristina Broliden
Biology of Reproduction. 2015 Mar; 92(3):68
- III. **Expression profiles of antimicrobial peptides in the genital tract of women using progesterone intrauterine devices versus combined oral contraceptives**
Andrea Introini, Tove Kaldensjö, Taha Hirbod, MARIA RÖHL, Annelie Tjernlund, Sonia Andersson and Kristina Broliden
American Journal of Reproductive Immunology. 2014 Nov; 72(5):475-84
- IV. **Intact tissue microenvironment and HIV target cell expression in the ectocervical epithelium of HIV-exposed seronegative sex workers**
MARIA RÖHL, Annelie Tjernlund, Julie Lajoie, Gabriella Edfeldt, Genevieve Boily-Larouche, Muhammad Asghar, Julianna Cheruiyot, Makubo Kimani, Joshua Kimani, Julius Oyugi, Keith R. Fowke*, Kristina Broliden*
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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AMP	Antimicrobial peptides
APC	Antigen presenting cell
ART	Antiretroviral therapy
BV	Bacterial vaginosis
CCR	CC chemokine receptor
CD	Cluster of differentiation
COC	Combined oral hormonal contraceptives
CTL	Cytotoxic T lymphocytes
CVS	Cervicovaginal secretions
CXCR	CXC chemokine receptor
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FGM	Female genital mucosa
FSW	Female sex workers
ART	Antiretroviral therapy
HC	Hormonal contraceptive
HESN	HIV-exposed seronegative
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HSV	Herpes simplex virus
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
LC	Langerhans cell

MHC	Major histocompatibility complex
MR	Mannose receptor
NHP	Non-human primate
NK	Natural killer
PCR	Polymerase chain reaction
pIUD	Progesterone-based intrauterine device
PrEP	Pre-Exposure Prophylaxis
PRR	Pattern recognition receptor
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
SIV	Simian Immunodeficiency virus
STI	Sexually transmitted infection
TCR	T cell receptor
TEM	Effector memory T cells
TRM	Tissue-resident memory T cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
UBC	Ubiquitin C

1 INTRODUCTION

1.1 The human immunodeficiency virus (HIV)

The first medical report on HIV was published in 1981 and described four homosexual men who suffered from lymphopenia and opportunistic infections such as pneumocystis pneumonia, a disease normally associated with severe immunosuppression (1). This was followed by reports of other cases, in homosexual men, with rare opportunistic diseases together with symptoms including fever and weight loss (2, 3). The aggressive new disease was characterized by severe immunosuppression, therefore named acquired immunodeficiency syndrome (AIDS) (1982) (4) and it took approximately two years to identify the virus, that would later be known as HIV-1, as the cause of AIDS (5). This discovery resulted in the Nobel prize in 2008 (6). In 1986 another type of HIV, named HIV-2, was discovered (7) and is mainly found in West Africa. HIV-1 is responsible for the HIV epidemic worldwide and will be referred to as HIV in this thesis.

HIV is derived from the Simian Immunodeficiency virus (SIV), which infects non-human primates (NHP) (8, 9). The natural hosts such as African green monkeys and sooty mangabeys, do not develop any clinical symptoms, while non-natural hosts such as monkeys of Asian origin develops a disease in resemblance to human AIDS (10). HIV belongs to the lentivirus genus and the retrovirus family, and retroviruses hold the unique ability to reverse the pathway of genetic information progress using the enzyme reverse transcriptase so that it passes from RNA to DNA. The viral DNA are then inserted to the host cells' DNA, with use of the viral enzyme integrase, and thereby a chronic infection is established.

1.1.1 Clinical course of HIV infection

During initial HIV infection, the plasma viral load increases exponentially and peaks over 6 logs in approximately two weeks after when it is first detected in blood (11). This progress is followed by a sharp decrease of viremia to a viral set point level of two logs lower a month later (12). The clinical manifestation of the first one to three weeks with high plasma viral load includes symptoms similar to those of glandular-fever, including fever, tonsillar hypertrophy and myalgia (13). This stage is referred to as the “acute/early phase”, with leucopenia, a significant loss of cluster of differentiation (CD) 4 receptor expressing T cells and a high viral load (13). CD4⁺ T cells are the primary target cells for HIV infection and the CD4⁺ T cell count together with plasma viral load are essential markers used for monitoring the progression of HIV disease. The early phase of HIV infection is characterized by a massive depletion of CD4⁺ T cells (14). This process predominantly occurs in the gastrointestinal tract, and to a lower extent in peripheral blood, lymph nodes and cervix (15). Two factors are suggested to be the main causes for the ultimate cell depletion over time; the progressive CD4⁺ T cell failure mediated by the virus itself and the high immune activation

(16). Early initiation of antiretroviral therapy (ART) treatment restores to a certain extent the CD4⁺ T cell levels, although not as high levels as prior to infection (17). The acute phase is followed by the “clinical latency phase” characterized by the absence of clinical symptoms and a low viral replication of HIV. With treatment, this phase can continue for a long time, although with no treatment it continuous for an average of 10 years (15). The clinical latency phase may be followed by development of AIDS if no treatment for HIV infection is initiated. This development involves a decline in the number of CD4⁺ T cells (lower than 200 cells/mm³), the loss of vital immune function, progressive impairment of cellular immunity, and thus increased susceptibility to opportunistic infections, such as *Pneumocystis carinii* pneumonia and an increased risk of cancer, including *Kaposi’s sarcoma* (1, 3, 16).

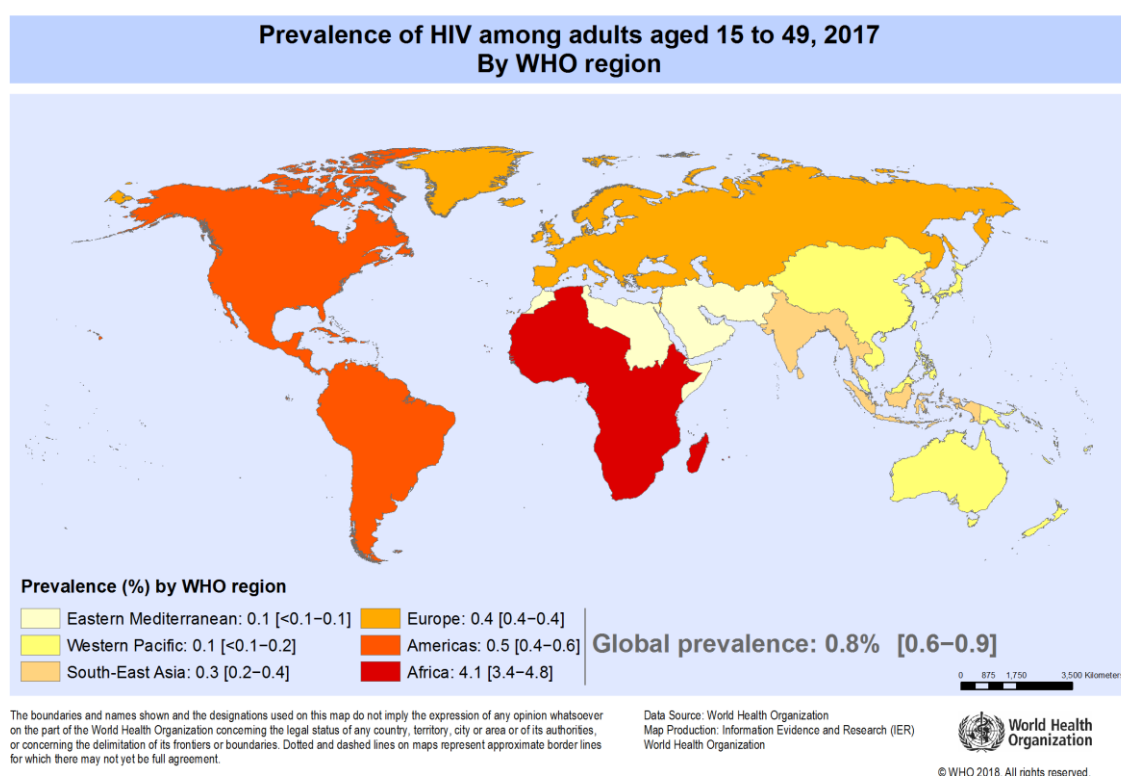


Figure 1. The global HIV-1 prevalence latest report 2017 according to WHO. Generalized by world major regions, including Eastern Mediterranean, western Pacific, South-East Asia, Europe Americas and Africa. HIV-1 prevalence per region was predicted based on representative populations within that region.

1.2 Global HIV distribution

The prevalence of HIV during 2017 was reported to be around 36.9 million people (18) (**Figure 1**). African countries are suffering from the HIV epidemic and especially the Sub-Saharan part of Africa is considered to be the most influenced region, with 66% of all global new HIV infections according to the latest report (18). In the same year, there was an estimation of 25.6 million HIV-seropositive people living in Sub-Saharan Africa (19). Major barriers to testing include stigma and discrimination, inaccessibility of services, and low awareness of the benefits of testing and early treatment (20). The dominant route of HIV transmission in Africa is heterosexual intercourse, which is in contrast to both Europe and

USA. E.g. in Western Europe the predominant route of HIV transmission is sex between men, while in Eastern Europe drug injections and heterosexual transmission are the most common routes of transmission (18). HIV transmission in the European regions has not drastically declined the last 10 years, despite the development and increased availability of ART, as well as extensive public health efforts (18). The estimated number of new HIV infections in Central Asia and in Eastern Europe during 2016 was 190.000, which was alarmingly 60% higher as compared to year 2010 (18).

1.2.1 HIV prevalence among key populations: sex workers

UNAIDS considers sex workers, men who have sex with men, transgender people, people who inject drugs and prisoners and other detained people as the main key population groups who are especially vulnerable to HIV and commonly lack adequate access to services. In 2016, excluding the Sub-Saharan Africa region, key populations and their sexual partners accounted for 80% of all new HIV infections. Even in Sub-Saharan Africa, key populations accounted for 25% of new HIV infections during this year (18). Sex workers are at very high risk of acquiring infection, and female sex workers (FSW) are reported to have a 13 times higher HIV prevalence than the general population (21). This is particularly the case in countries where the HIV prevalence is very high such as in South Africa, where the HIV-prevalence rates in young women in Umlazi Township rose from less than 1% at age 15 to 66% at age 23 (22).

In this thesis, we have focused on FSW in Nairobi, Kenya. In Kenya, the HIV-prevalence is 5.4% in adults between the ages of 15-49 years as compared to 0.8% globally (18). The prevalence of HIV among FSW in Kenya is approximately 30% and furthermore, about 50% of HIV-positive Kenyan FSW are not aware of their HIV status, despite national STI-testing programmes (23). This results in severe consequences such as delays in access to treatment and healthcare, as well as continuous HIV transmission (23). There are several factors that contribute to increased vulnerability to HIV transmission that could be avoided by assessment of issues present in key populations, such as FSW, together with legal measures. For example, unprotected sex, sexual violence, discrimination as well as social and economic factors contribute to the increased susceptibility to HIV transmission in FSW (24). It is implied that elimination of sexual violence alone could prevent 17% of HIV infections in FSW and their clients. Moreover, decriminalization of selling sex, but not buying sex, could prevent 33% of HIV infections in FSW and their partners during the next decade (25). By focusing on improvement in these areas, the HIV transmission in vulnerable groups may decrease and subsequently decrease the overall HIV transmission.

1.3 HIV treatment and challenges

The most important step in the process of limiting the HIV epidemic has been the introduction and development of ART (26). The access of ART has significantly increased over the years; in 2001, only 1 million HIV seropositive people had access to ART, as compared with an estimated of 20.9 million in June 2017 (18). During the establishment of ART and the following reduction of HIV transmission, the Pre-Exposure Prophylaxis (PrEP) treatment could be implemented in risk groups who were at a high risk of acquiring infection (27, 28). The PrEP treatment is a daily intake of a pill containing two different medicines, tenofovir and emtricitabine. According to the federal guidelines by US Centers for Disease Control and Prevention (CDC), PrEP should be administered to risk groups such as FSW and HIV seronegative individuals who have an ongoing sexual relationship with an HIV-positive partner.

Besides the positive outcomes of ART, there are issues associated with the life-long treatment including side-effects such as lipodystrophy and insulin resistance (29, 30). Furthermore, antiviral drug resistance is prevalent, and develops under suboptimal therapeutic conditions due to for instance patient incompliance or self-medication along the increased access to ART (31). However drug resistance can also appear even with successful viral suppression (32). Viral reservoirs furthermore act as sanctuary sites, as tissues or cells are inefficiently targeted by the antiretroviral drugs, and thus continuous HIV-replication occurs in various reservoirs under antiretroviral therapy (29). Another issue is the inevitable consequences of an increased economical demanding due to the increased number of individuals receiving ART.

1.3.1 Future goals

UNAIDS is today reaching towards the new global goals in the process of defeating the HIV epidemic. **i)** To achieve the 90-90-90 treatment target by year 2020. This goal is that in 2020, 90% of all HIV-infected people will be aware of their HIV status, 90% of all HIV- infected people who know their HIV status will receive ART, 90% of people with ART will show suppressed viral loads (90-90-90) (21, 33), **ii)** Less than 500 000 new HIV infections will occur annually and the global HIV- related deaths will decline to fewer than 500 000 in 2020. Ultimately, the main goal is that the HIV epidemic will be ended by year 2030 (21).

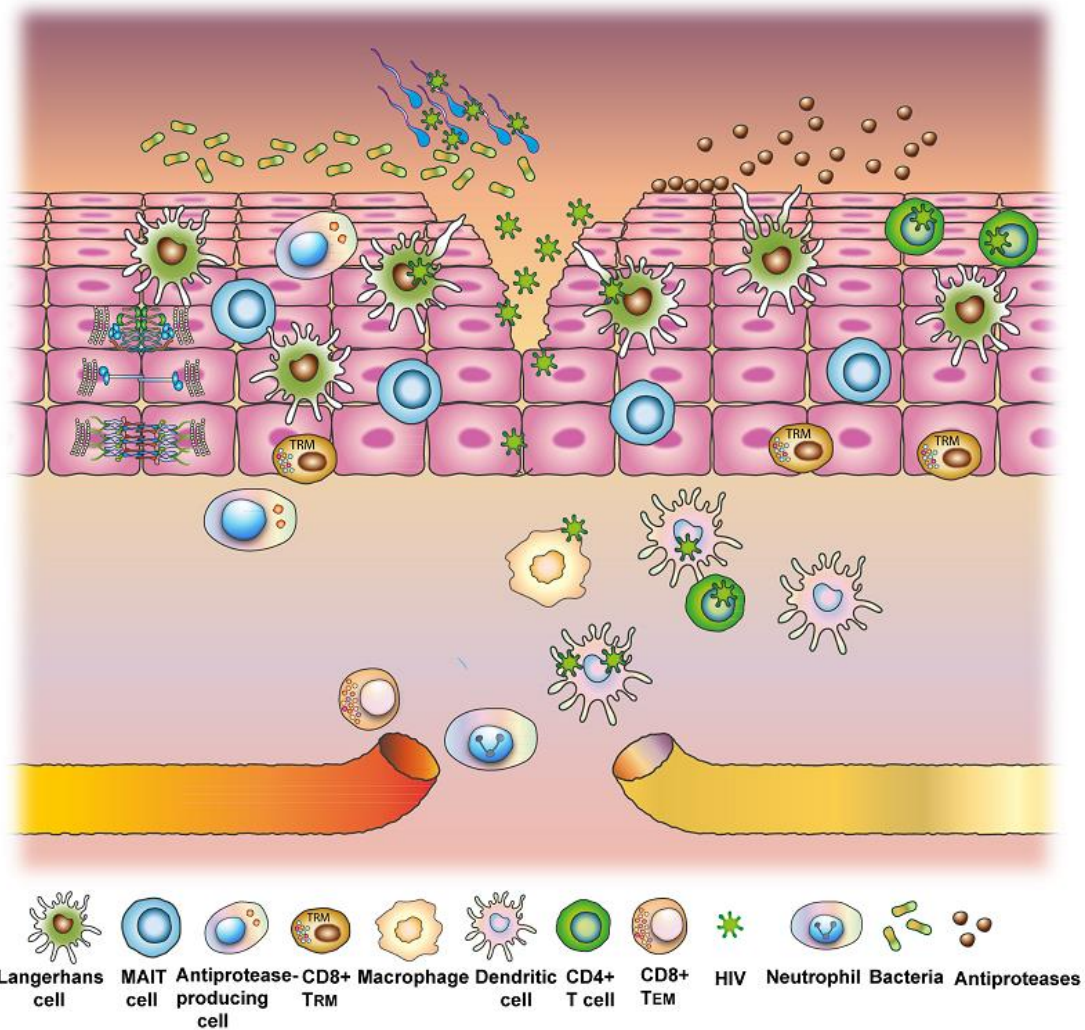


Figure 2. Schematic illustration of the suggested mechanism for HIV heterosexual transmission across the female genital mucosa. The female genital mucosa is harboring HIV target and effector cells, as well as epithelial tight junction proteins, which are protecting against invading pathogens like HIV. The epithelial linings are covered by genital secretions containing hormones, microbes and innate proteins also important in HIV transmission. Micro-breaks in the epithelium are thought to facilitate entrance of HIV.

1.4 HIV transmission and mucosal immune responses to HIV

Sexual HIV transmission is the most common route of transmission followed by mother – to – child transmission (vertical transmission) and then blood-borne infection (21, 34). The main target sites for sexual HIV transmission are the mucosal tissues of the foreskin, cervix and rectum (35).

To initiate infection, the virus needs to reach the HIV target cells which are located both in the epithelium and in the underlying submucosal tissue (36) (**Figure 2, Figure 3**). The degree of virus infiltration is influenced by the phenotype and concentration of virus and the efficiency of the protective mucosal barrier functions.

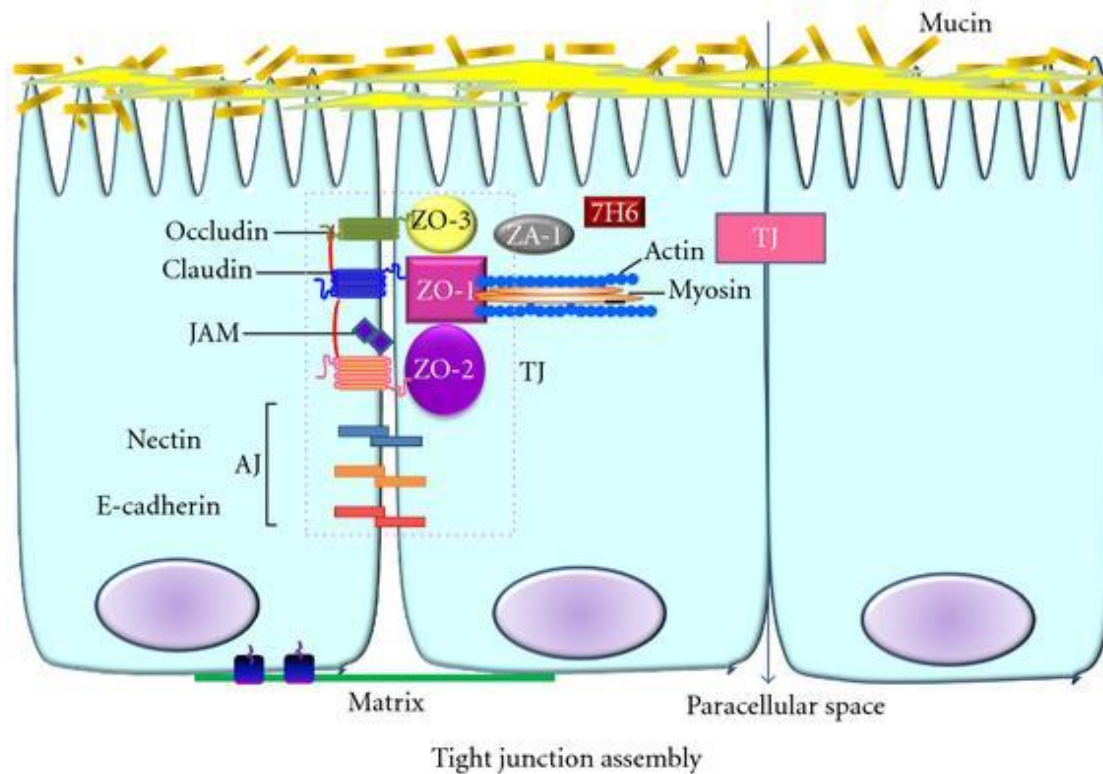


Figure 3. A model of the tight (TJ) and adherens junctions (AJ) in an epithelial cell. The TJ consist of transmembrane proteins (occludins, claudins, and JAMs) linked to an actin cytoskeleton via cytoplasmic ZO (zonula occludens) proteins and ZO-1 binds to actin. The AJ are composed of the nectin and the E-cadherin-catenin system. TJs and AJs form the apical junctional complex, which is linked to the actin cytoskeleton network. Reprinted with permission (37).

HIV enter their target cells by binding its viral envelop protein gp120 to the CD4 molecule on the cell surface of the target cells. This induces a conformational change which promotes binding of the viral envelop protein gp41 to one of the two co-receptors, CCR5 or CXCR4 chemokine receptor 4 (CXCR4) that subsequently induces insertion of the distal tips of gp41 into the cellular membrane and fusion occurs between the viral- and cellular membranes. The initial HIV transmission predominantly occur in a CCR5 dependent way (38) and the initial infection events generate a small population of founder cells within the mucosal tissue. This founder population expands and the virus disseminates to draining lymph nodes, with the help of antigen presenting cells (39, 40). Subsequently, the infection is spread systemically and the main HIV replication occurs in the lymph nodes (41).

The HIV target cells in the cervical mucosa include CD4 expressing T cells, dendritic cells (DCs) and macrophages (42, 43). Langerhans cells (LCs), is a subset of DCs localized in the ectocervical epithelium, expected to be among the earliest cells to encounter the virus and which may further pass the virions to CD4⁺ T cells (44) that are thought to be the main target cells for HIV (39, 45, 46). LCs can bind to HIV in two different ways; either through the CD4/CCR5 receptors (same as the CD4⁺ T cells) or via the Langerin pathway (47). Langerin

is a C-type lectin receptor which binds specifically to the high mannose glycan present on HIV gp120, and this may lead to internalization of the virus into Birbeck granules followed by viral degradation (48, 49) (**Figure 4**). Birbeck granules are langerin-positive organelles exclusively present in LCs and have been reported to be linked to the lysosomal degradation pathway in LCs. However, the function of Birbeck granules and how it contributes to limiting HIV-1 infection remains to be determined. A recent study by Pena-Cruz *et al* reported that vaginal LCs do not harbor Birbeck granules unlike other blood- and tissue-derived LCs (46).

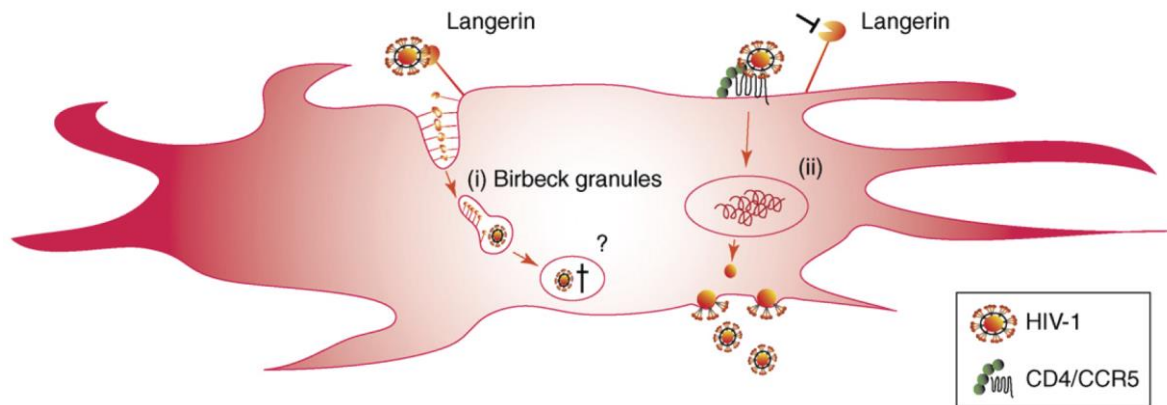


Figure 4. LCs interaction with HIV-1. Langerin is a receptor for HIV-1 on LCs and internalizes HIV-1 through the Birbeck granules (i). Langerin interaction with HIV-1 results in HIV-1 degradation, viral clearance and inhibition of HIV-1 transmission. However, the specific function of Birbeck granules is not fully established (ii) A block of Langerin function through drugs, co-infections or mutation may lead to LCs transmitting HIV-1 efficiently to T cells (right) through infection of the LCs. Adapted with permission (50).

As mentioned above, the CD4⁺ T cells are thought to be the main target cells and they are abundant in ectocervical mucosa, where they are distributed in the epithelium and the underlying submucosa (43). More recently, studies have implied that the $\alpha 4\beta 7$ - and $\alpha 4\beta 1$ integrin expressing CD4⁺ T cells and the interleukin (IL) 17 producing CD4⁺ T cells (Th17) are more susceptible to HIV infection as compared to other CD4⁺ T cell subsets (51, 52). Cervical Th17 cells are furthermore depleted during early HIV infection, in concordance with the finding of the preferential infection of this cell type (53). DCs situated in the submucosa can facilitate CD4⁺ T cell infection through the C-type lectin receptors DC-SIGN and mannose receptor (MR) that binds to HIV gp120 (54-56). Additionally, CD4⁺CCR5⁺ macrophages are another cell population present predominantly in the submucosa close to the basal membrane, and have been shown to be productively infected by HIV *ex vivo* (57).

However, in order for the virus to infect their target cells they have to gain access to the tissue which are protected by several mechanisms. Mucus and the commensal microbiota make up a first line of defense, which hinder pathogens to enter the frontline tissue/epithelium. Soluble factors belonging to the innate and adaptive immune system are present in the mucus/secretions as well as within mucosal tissue and constitutes another line of defense that prevent infections (58). Among these immunologic mediators are a heterogeneous group of small proteins displaying microbicidal activity against a broad range of pathogens, generally

referred to as antimicrobial peptides (AMPs) (59). AMPs are constitutively produced by epithelial cells and leukocytes in the female genital mucosa (FGM) (60, 61). AMPs include defensins, comprising of human neutrophil peptides (HNP) 1-3, or α -defensins 1-3, and β -defensin (BD) 2, human cathelicidin antimicrobial peptide (hCAP) 18/LL-37, and the acidic proteins secretory leukocyte protease inhibitor (SLPI) and trappin-2/elafin. These factors have different mechanisms of action, but they have all been demonstrated to inhibit the infectivity of a number of microorganisms *in vitro*, in particular HIV-1 (59).

The epithelium itself also makes up a line of defense; it contains the epithelial junction protein complex, a network of proteins that constitutes a selective barrier. It consists of tight junction proteins, adhesion proteins and desmosomes that allow penetration of essential molecules whereas it prevents pathogens to enter the tissue. *Ex vivo* studies in cervical explant models have shown that HIV virions can infiltrate up to 50 μ m of the intact ectocervical epithelium (62) and inflammation can affect this barrier so that pathogens, including HIV, can more easily gain access to their target cells in the mucosal tissue (36, 63, 64). Furthermore, sex hormones can also affect this barrier and facilitate the viral access to HIV target cells (36). However, the genital mucosa is also populated by cells belonging to the innate and adaptive immune system, which adds another layer of protection against HIV infection. Innate lymphoid cells (ILC), including traditional natural killer (NK) cells are suggested to mediate early control of HIV infection (65, 66) while HIV-specific effector CD8⁺ T cells are important players of the adaptive immune response in HIV-infected individuals (67, 68).

1.5 HIV susceptibility factors

The risk of vaginal male- to- female HIV-1 transmission is low, about 0.08-0.3 % per unprotected sexual intercourse, as compared to anal sex where the risk is about 18 times higher (69, 70). Proven modulating factors are sexually transmitted infections (STIs) such as genital herpes caused by herpes simplex virus type 2 (HSV-2). Women who are HSV-2 infected are approximately 9 times more likely to acquire HIV as compared to HIV-2 seronegative women, and this is considered to partly be due to HIV entry through herpetic lesions and the increased counts of tissue residing CD4⁺ T cell seen during HSV-2 lesion events (71). Furthermore, sex hormones such as progesterone can also facilitate the viral access to HIV target cells. During the progesterone-high secretory phase of the menstrual cycle, uterine cytotoxic T lymphocyte (CTL) activity and NK cell cytotoxic activity are suppressed, whereas innate components are upregulated and the frequency of CD4⁺ expressing T cells are increased (36, 72). The balance in the microflora can also affect the risk of acquiring HIV. The balance is normally characterized by dominance of *Lactobacillus* species which is associated with lower HIV prevalence, while dysbiotic women with clinical bacterial vaginosis (BV) are at higher risk of HIV acquisition (73, 74). BV is associated with elevated levels of inflammatory cytokines and reduced levels of factors important for maintenance of mucosal barrier integrity, potentially explaining why BV contributes to

increased HIV acquisition (75, 76). The inflammatory process itself can affect the genital mucosal barrier so that pathogens, including HIV, can gain access to their target cells in the mucosal tissue (36, 63, 64). Furthermore, other STIs including the presence of *N. gonorrhoeae* and *Chlamydia trichomonas*, contribute to a pro-inflammatory milieu in the genital mucosa and is associated with increased risk of HIV acquisition and transmission (64, 77, 78). Thus, the composition of the genital microflora and STIs along with the mucosal innate immune system and the robustness of the genital epithelial barrier could further contribute to increased host susceptibility to infection (79).

1.5.1 Herpes simplex virus 2 (HSV-2) and HIV susceptibility

HSV-2 is one of the most common STIs of the genital skin and mucosa. More than 50% of all people living in Sub-Saharan Africa were reported to be seropositive for HSV-2 in 2014 (80), which are the highest levels of HSV-2 infection in the world. The exact levels vary however from country to country within this continent. This region also displays a high HIV prevalence (**Figure 1**). HSV-2 infection has been strongly associated with HIV infection (81-83) and appears to increase both HIV susceptibility and the potential for a co-infected individual to transfer HIV to sexual partners (81, 83).

During primary infection, HSV-2 infects genital epithelial cells and is then transported via sensory nerves to the sacral root ganglion, where lifelong latency is established. Intermittent genital shedding of HSV-2 in the immunocompetent person is frequent, with or without symptoms (84). In situ analysis of genital skin biopsies from HSV-2 lesions have revealed that cellular infiltrates and local inflammation persisted for months after healing, even with daily antiviral therapy (85). Genital skin lesions and activation of the mucosal immune response at time of local reactivation increase the risk of sexual transmission of the virus, as well as the risk of acquiring other genital infections, including HIV (85-87). The persistent mucosal inflammation of the human skin samples, caused by HSV-2 infection, including upregulation of the HIV co-receptors DC-SIGN and CCR5, may partly explain the inability of the antiviral HSV-2 therapy to reduce acquisition of HIV (85, 88, 89).

1.5.2 Effects of progesterone on HIV susceptibility in female genital mucosa

The female sex hormone progesterone has considerable effects on the genital mucosa including susceptibility to STIs (90, 91). In NHP-models it is clear that high progesterone levels significantly increase the susceptibility to SIV, as well as promote viral shedding in animals already infected with SIV (92). The widespread use of progesterone-based hormonal contraception (HC) is a much-appreciated method of birth control. There are different types of progesterone-based HCs such as progesterone intrauterine device (pIUD) containing the progestin levonorgestrel, combined oral contraceptives (COC) consisting of an estrogen (estradiol) and a progesterone (progestin) component and the injectable depot

medroxyprogesterone acetate (DMPA) containing a progestin component. Progesterone-based HC, and especially the high progesterone injectable DMPA, has been shown to increase susceptibility to primary HIV infection as well as HIV shedding and transmission in already infected women (36, 93-95). The exact mechanism of this is not yet established, although progesterone-based HC have been associated with a thinning of the cervical epithelium and an increase in migration and activation of DC subsets in cervical explant models which theoretically may lead to increased HIV susceptibility (96-98).

1.6 HIV Exposed Seronegative (HESN) women

While some individuals become infected by HIV, others persist uninfected despite continual exposures and are defined as HIV-exposed seronegative (HESN). Reports from this group have included FSW, HIV serodiscordant couples, intravenous drug users, hemophiliacs and infants born to HIV-infected mothers (99, 100). Studying the HESN group provides a unique opportunity to investigate natural protection (relative resistance) against HIV and examples of factors associated with HESN FSW are outlined in **Table 1**.

Table 1

Examples of factors associated with the HESN status in female sex workers

Factor	Correlation with HESN	References
Genetic loci polymorphisms		
Toll-like receptor 3 (TLR3) polymorphism	Reduced frequency	Sironi <i>et al.</i> (2012)
Complement receptor 2 (CR2) polymorphism	Higher frequency	Herrero <i>et al.</i> (2015)
Complement component 4 binding protein alpha (C4BPA)	Higher frequency	Herrero <i>et al.</i> (2015)
Myxovirus resistance 2 (MX2)	Higher frequency	Sironi <i>et al.</i> (2014)
Endoplasmic reticulum aminopeptidase type 2 (ERAP2)	Reduced frequency	Biasin <i>et al.</i> (2013), Cagliani <i>et al.</i> (2010)
Apolipoprotein B editing catalytic polypeptide (APOBEC 3H)	Higher frequency	Cagliani <i>et al.</i> (2011)
β -Defensin 1 (DEFB-1)	Higher frequency	Zapata <i>et al.</i> (2008)
Immune determinants		
Interleukin-1 beta (IL-1 β)	Lower levels	McLaren <i>et al.</i> (2010)
Interleukin-6 (IL-6)	Lower levels	McLaren <i>et al.</i> (2010)
Interferon gamma (IFN γ)	Lower levels	Pala <i>et al.</i> (2013)
Activated T cells	Lower proportion	Card <i>et al.</i> (2013)
T regulatory cells	Higher levels	Thibodeau <i>et al.</i> (2017)
Chemokine (C-C motif) ligand 9 (CCL9)	Lower levels	Lajoie <i>et al.</i> (2012)
Interleukin-1 alpha (IL-1 α)	Lower levels	Lajoie <i>et al.</i> (2012)
Chemokine (C-C motif) ligand 10 (CCL10)	Lower levels	Lajoie <i>et al.</i> (2012)
Innate antiinflammatory protease serpin	Higher levels	Aboud <i>et al.</i> (2014), Gonzalez <i>et al.</i> (2015)
Innate antiinflammatory protease cystatin	Higher levels	Aboud <i>et al.</i> (2014)
Systemic CD4+CCR5+ T cells	Lower proportion	Jaumdally <i>et al.</i> (2017)
HIV-1 neutralizing immunoglobulin A (IgA) antibodies	Higher levels	Devito <i>et al.</i> (2000), Hirbod <i>et al.</i> (2008)

In our specific study cohort in Nairobi, Kenya, we study HESN FSW, with HESN defined as women who are and have been active in sex work for at least 7 years. It has been speculated that these HESN women display low inflammation/immune activation status in their genital mucosa despite being sex workers (101-105). Furthermore, studies on a Beninese cohort of FSW also support maintenance of low-inflammatory conditions in the FGM of HESN individuals (106-109).

Considerable genetic and immunological associations to the HESN phenotype have been reported in our cohort, such as reduced gene expression in the TLR-signaling pathway crucial for T cell activation (110, 111) as well as lower proportion of activated T cells and higher proportion of T regulatory cells (112) in the circulation. Furthermore, lower levels of inflammatory cytokines (103) and higher levels of innate anti-inflammatory antiproteases have been detected in the genital tract in the same cohort (113). However, the mechanisms of protection against infection associated to the HESN phenotype remains to be established and are probably both multifactorial and individual.

2 AIM

The main purpose of this thesis was to investigate how asymptomatic HSV-2 infection, progesterone-based HC, and relative resistance against HIV affect the local FGM milieu in order to better understand HIV susceptibility factors.

Specific aims:

Paper I. To investigate how HSV-2 seropositivity correlates with markers of the genital epithelial barrier and mucosal immune response in an asymptomatic male population in Kenya.

Paper 2. To investigate how the use of progesterone-based HC affects the genital epithelial barrier and mucosal HIV receptor expression in healthy Swedish women.

Paper 3. To investigate how the use of different HCs affect the production of AMPs in different compartments of the FGM including secretions and tissue.

Paper 4. To investigate if epithelial thickness and quantity/localization of HIV target cells in ectocervical epithelium were linked with the relative resistance against HIV, which are associated with a HESN phenotype

3 MATERIALS AND METHODS

3.1 Study populations and sample collection

Samples from the following cohort were used in this thesis.

The Kisumu cohort (Kenya): Foreskin tissue biopsies were obtained from men undergoing elective circumcision in a randomized, placebo-controlled clinical trial conducted in Kisumu, Kenya, through the Universities of Nairobi, Illinois and Manitoba Collaborative Research Project. The unblinded trial included two arms: the circumcision arm and the delayed circumcision (control) arm to examine whether male circumcision reduced HIV incidence (114). For the substudy in **Paper I**, samples were randomly selected and divided into two study groups according to HSV-2 serological status. Inclusion criteria were absence of clinical or laboratory signs of genital ulcers at time of circumcision and that the participants were HIV seronegative, sexually active, 18-24 years old, resident of Kisumu district, no plans to move for at least two years and haemoglobin 90g/L or more. Exclusion criteria were foreskin covering less than half of the glans, haemophilic or other bleeding disorder, high prothrombin time index and other medical condition contraindicating surgery. For our substudy, equal numbers of specimens were thus randomly selected from each study group and matched for age (18–20 and 21–24 years). Screening for sexually transmitted diseases included a history of discharge and previously diagnosed STIs, a clinical examination 2 weeks before and at the time of circumcision, as well as laboratory analysis of STIs (114). Serum specimens were tested for HSV-2 antibody (Kalon HSV-2 IgG ELISA, Kalon Biological Limited, Aldershot, UK) using the manufacturer's recommended cut-off (115). The tissue biopsies were collected by dissection following surgery, and snap frozen and subsequently stored at – 80°C.

The Karolinska cohort (Stockholm): Cervicovaginal samples were collected from premenopausal volunteers enrolled at the Karolinska University Hospital. Laboratory analysis of STIs was performed and inclusion criteria was HIV seronegative status. Women using systemic immunosuppressive therapy or with a history of cervical pathology were excluded.

Ectocervical tissue biopsies were collected from women using pIUD, COC or no HCs and were included for the studies in **Paper II**. Two biopsies from the superior portion of each donor's ectocervix were collected by an experienced gynecologist. One of the biopsies was immediately frozen in liquid nitrogen and subsequently used for in situ staining, and the other biopsy was preserved in RNAlater and used for quantitative real-time polymerase chain reaction (qPCR) experiments.

Furthermore, ectocervical tissue samples and cervicovaginal secretions (CVS) were obtained from women using pIUD or COC in **Paper III**. Ectocervical tissue biopsies were collected as described above. The CVS samples were collected by using a cotton-tipped swab that was

rotated 360° in the ectocervical os and a second swab to collect secretions from the posterior vaginal fornix.

The Pumwani cohort (Kenya): In **Paper IV**, the study subjects were recruited from the Pumwani Commercial Sex Worker Cohort in the Majengo clinic in Nairobi, Kenya. Inclusion criteria were that the women should be active in sex work, uterus and cervix present, 18 to 50 years of age and being premenopausal, willing to undergo cervical biopsies and undertake a month-long period of sexual abstinence, having a regular menstrual cycle, no hormonal contraceptive use, not pregnant or breastfeeding and in general good health. Laboratory analysis of STIs was performed. The cohort includes two study groups: HESN women, who had been involved in sex work for seven years or more and HIV-seronegative women who had been involved in sex work for three years or less (new negatives, NN) which served as the control group. Ectocervical tissue biopsies was collected, as described above for the Karolinska cohort, by an experienced gynecologist at the Kenyatta National Hospital, Nairobi, Kenya.

3.1 Ethical considerations

All study participants were provided with written and oral information about the studies. Written informed consent was obtained from all individuals and all studies were reviewed and approved by the Regional Ethical Review Board of Stockholm (Stockholm, Sweden) (**Paper I-IV**), and additionally approved by the Kenyatta National Hospital ethics and research committee, the University of Illinois at Chicago institutional review board and the University of Manitoba biomedical research ethics board (**Paper I**), and approved by the ethics boards at Kenyatta National Hospital (Nairobi, Kenya) and University of Manitoba (Winnipeg, Canada) (**Paper IV**).

Collecting genital samples is inevitably associated with ethical concerns and a gynecological examination can generally be followed by a feeling of there being an infringement of person integrity. Thus, only experienced specialists were involved in carrying out the examinations and in the collection of the samples included in our studies, in order to avoid any negative experiences as far as possible. During biopsy sampling a light bleeding can occur and a certain amount of discomfort can be experienced. The cervical biopsy sampling included in all papers has been evaluated and considered to be a safe and well-tolerated method (116). Regarding male circumcision, it is a well-established and safe method reducing HIV susceptibility and also the incidence of urinary tract infections and penile cancer according to UNAIDS (117). Men in Kenya who underwent extended follow-up exhibited sustained reductions in HIV incidence of 64% at five and a half years after the circumcision (117).

Research including the Pumwani cohort involves our Canadian research collaborators who established the cohort more than 30 years ago. They have a strong community outreach program including regular information meetings for study volunteers and program peer leaders.

All participants were provided with HIV/STI prevention counselling, condoms, family planning assistances, treatment of STIs, medical care for acute and chronic illnesses, access to adequate diagnostic testing and referral for specialist consultant and/or hospitalization at Kenyatta National Hospital if required. Ectocervical tissue biopsies were obtained from FSWs and sampling of the ectocervical biopsies may raise concerns regarding the increased HIV susceptibility in this population. Therefore, all study participants were asked to abstain from vaginal sex during two weeks after the procedure and received monetary compensation equivalent to the expected loss of income. On-site detection of prostate-specific antigens indicated unprotected intercourse, and this detection has been recently evaluated in our cohort as a tool for limiting HIV exposure in studies requiring biopsy sampling (118).

3.3 Methods

3.3.1 Quantitative real-time PCR

Quantitative real-time PCR has many objective advantages over other PCR analysis such as speed, broad dynamic range of target DNA quantitation, and reduction of contamination although it is not necessarily more sensitive. mRNA expression of the genes of interests in the ectocervical and foreskin biopsies was assessed with qPCR as previously described (119). qPCR was used in **Paper I-III**. RNA was obtained from the RNAlater-stored biopsies by the commercially available RNeasy kit (Qiagen), according to the manufacturer's protocol, and the RNA was subsequently converted into complementary (c) DNA using a Superscript II reverse transcriptase kit (Life Technologies). cDNA of the genes of interest was amplified, detected and quantified by the ABI PRISM 7700 system. Amplification of ubiquitin C (UBC) was used as an endogenous control. Ct values for target cDNA were normalized to UBC and fold change of the target genes was calculated using the $2^{-\Delta\Delta C_T}$ equation.

3.3.2 Enzyme-linked immunosorbent assay (ELISA)

The Enzyme Linked ImmunoSorbent Assay (ELISA) is a technique that has been used for over 40 years. As the technology developed the assay itself has become much more sensitive and is a good way to measure antigen levels in complex liquid sample. ELISA is quick and convenient, and antigens of very low or unknown concentration can be detected since capture antibody only grabs specific antigen. However, limitations are that only monoclonal antibodies can be used as matched pairs, which may be difficult to find, and enzyme/substrate reaction is short term so the optical density must be read as soon as possible. In **Paper III**, the concentration of AMPs in CVS samples was measured. The specific concentration of proteins including HNP1-3, LL-37, trappin-2, BD-2 and SLPI was measured by ELISA according to the manufacturer's instructions.

3.3.3 *In situ*-based imaging analysis

There are logistic difficulties of collecting tissue samples, in particular from the genital mucosa from individuals who are at high-risk of infection or from HIV-endemic areas. Genital tissue biopsies can be safely collected from study participants (116) and can be stored at -80°C for a long period. Microscopy-based *in situ* methods allow analysis of properties of single cells in small tissue samples and have an excessive visual advantage as compared with other cell-based methods such as flow cytometry. The exact anatomical cell localization and distribution within the tissue can be assessed by *in situ*-based analysis of tissue samples, which is of importance to understand essential component of cell-mediated immunity (120). Nevertheless, there are limitations associated with microscopy-based methods. The methods are dependent on specific antibodies which may not be available and limit the assessment of several markers at the same time, restricting phenotypical and functional cell evaluation. Furthermore, autofluorescence from the tissue, which often varies from different tissue compartment as well as from different individuals, is an issue that needs to be considered. Furthermore, a restriction is data acquisition and analysis. Manual cell counting is subjective and time consuming, and may thus be inappropriate for analysis of large-scale studies. Opposed to manual cell counting, automated cell counting gives greater precision, less variation and is less time-consuming. Nevertheless, the automated software is often inferior in addressing inter-individual variation of biological material as compared with manual analysis performed by an expert.

In **Paper I-III**, manual image analysis was performed. In **Paper IV**, the automated image analysis software CellProfiler was used, which allows high-throughput image analysis and quantification of different immunological parameters in tissue sections (121, 122). CellProfiler was used for quantification of positive cells, detected with use of fluorescently labelled antibodies, in the ectocervical tissue sections.

In **Paper II** and **Paper III**, cells were visualized with a peroxidase-labelled streptavidin-biotin amplification method as compared with **Paper I** and **Paper IV**, where immune cells were stained with fluorescently labelled antibodies. Immunofluorescent staining allows visualization of several markers on a single cell more precise as compared to immunohistochemical staining due to the use of specific excitation and emission wavelengths. However, fluorochrome-based signals are usually not as stable (depending on the signal intensity and storage conditions) as compared to chromogen-based staining/signals. We thus scanned our immunofluorescent staining into digital images immediately after the staining was performed.

3.4 Statistical analysis

Paper I: The main objective was to assess the differences in HSV-2 seropositive men as compared to HSV-2 seronegative men. Categorical variables/clinical characteristics between

the study groups were compared by Pearson χ^2 test, and with Fischer's exact test when the numbers of the groups included the clinical parameters were less than 5. Continuous variables were analyzed by using unpaired two-tailed Mann-Whitney U test.

Paper II: In this study, the main objective was to compare the pIUD using women with the COC using women or with the group of women with noHC. Nonparametric comparisons were performed between the pIUD vs. COC and pIUD vs. NoHC and all immunological parameters were also compared between HPV positive and HPV negative women. Statistical significance between continues variables was assessed using the Mann-Whitney U test and Fisher's exact test was used for categorical variables.

Paper III: The two study groups consisted of women using pIUD and COC and Mann-Whitney test was used to compare continuous variables between the groups and the nonparametric Spearman's rank correlation test was used to assess linear association of clinical/continuous variables.

Paper IV: The main objective was to compare HESN women to NN women. Distribution of categorical variables was measured by Fisher's exact test. Mann-Whitney U- test was used for comparison of continuous variables between the groups. Further, multiple linear regressions were performed for adjusting statistical analysis for co-founding variables.

4 RESULTS AND DISCUSSION

Paper I. HSV-2 is one of the most common STIs of the genital mucosa and is associated with an increased susceptibility to HIV (81-83). The increased susceptibility to HIV might be due to the persistent inflammation that HSV-2 causes in the genital mucosa (89). We hypothesized that HSV-2 seropositivity was associated with genital mucosal inflammation and a disrupted epithelial barrier, even in asymptomatic individuals. We thus aimed to investigate if foreskin samples collected from asymptomatic HSV-2 seropositive individuals would differ in respect to immune activation status and epithelial junction markers as compared to HSV-2 seronegative individuals. Foreskin tissue samples were collected from men undergoing elective circumcision in Kisumu, Kenya and stratified into study groups based on HSV-2 serology. The two study groups were similar in sociodemographic, behavioral risk and STIs, except for the HSV-2 seropositive group which displayed a higher educational attainment and number of sex partners than the HSV-2 seronegative group.

qPCR was used to assess the overall immune activation status of the foreskin tissue samples. However, no differences were seen in the mRNA levels of the selected markers between the study groups. Namely, the mRNA expression levels of markers for cytokines (IL-1, IL-6, TNF- α , IFN- α , CCL5), immunoregulatory receptors (CCR5, HLA-DR and β 7), phenotypic cellular markers (CD4, CD3 and CD8,) the activation markers CD69, the CLR's Langerin, DC-SIGN and mannose receptor as well as of IgA were all similar across the study groups. Furthermore, the mRNA expression of the epithelial junction molecules E-cadherin, ZO-1 and occludin were also similar between the groups. However, the HSV-2 seropositive men had significantly lower mRNA expression of claudin-1 than the HSV-2 seronegative men. Nevertheless, no multivariate analysis was performed which would have been relevant in the study.

The epithelial junction proteins were further assessed by immunofluorescence staining for visualization of their tissue distribution. Claudin-1, as well as the other three epithelial junction proteins, was clearly expressed in the foreskin epithelium. However, the proteins showed an uneven distribution; some areas only had a few stained epithelial layers whereas other areas displayed several stained layers of thickness.

Subclinical and clinical inflammation is usually characterized by increased expression of cytokines, influx of inflammatory cells and reduced epithelial barrier integrity. This was however not confirmed by the results in this study. Here we found that no general inflammation, apart from a decreased expression of claudin-1 at mRNA levels were seen in the foreskin of asymptomatic HSV-2 seropositive individuals, as compared to HSV-2 seronegative individuals. A decreased expression of claudin-1 might indicate a less robust

genital epithelial barrier, which may affect the resistance of the mucosal barrier against HIV infection. This is exemplified by an isolated defect in only one of the epithelial junction proteins causing severe disease in individuals suffering from atopic dermatitis, in which results implicate that lower expression of Claudin-1 can enhance the spread of epidermal HSV-1 infections (123).

It would have been interesting to also quantify all the immune and epithelial junction markers at the protein level to see if these data would indicate a more inflammatory status in the asymptomatic HSV-2 seropositive individuals versus the HSV-2 seronegative individuals. We could also have included other inflammatory markers of interest such as interferon β and γ , as well as IP-10, macrophage inflammatory protein-1 β , IL-8, and monocyte chemoattractant protein-1. These may have been upregulated since they have previously been reported to be associated with HIV seroconversion in women (124). Thus, more markers need to be addressed in the future.

Unfortunately, there was no data on history of recurrent HSV-2 lesions. A less recurrent infection may not contribute to downregulation of epithelial junction proteins and increased levels of inflammation. Further, the foreskin tissue samples analyzed may not represent the exact localization where a previous lesion has occurred which may explain our results. The study participants could also have had a prior defect in claudin-1 expression either induced or genetically, which would be interesting to evaluate. A specific defect on the genital mucosal barrier in a highly vulnerable and relevant population could be of significance, since such defect may facilitate entrance of STIs including HIV. It would thus also be interesting to perform longitudinal studies to evaluate if claudin-1 is persistently down-regulated in the areas of HSV-2 lesions.

Paper II. The female sex hormone progesterone has considerable effects on the genital mucosa including susceptibility to sexually transmitted infections (90, 91). Today, there is an ongoing debate whether the widespread use of different progesterone-based HC affect HIV susceptibility in women. Hence, in this study, we wanted to investigate how the use of progesterone-based HC affects the genital epithelial barrier and HIV target cells in the genital mucosa. Difficulties in performing this type of study is that women on HC lack a normal menstrual cycle while women with noHC show great variabilities in hormone levels during the menstrual cycle. We thus included two control groups, COC (consisting of both estrogen and progesterone components) and noHC. The noHC group represents women at different stages of the menstrual cycle. Ectocervical biopsies were collected from premenopausal, healthy women using pIUD, COC or noHC. The three study groups were similar regarding age, relationship status and STIs, however, the pIUD group had significantly more sex partners during the previous year as compared to the noHC group.

In situ staining and image analysis were used to assess the total thickness of the ectocervical epithelium, from the basal membrane to the luminal border. While no statistical differences were seen between the pIUD vs. the COC study groups, the pIUD group had a significantly thinner epithelium compared to the noHC group. To further characterize the thickness of the different layers, the epithelium was stained for the adherence junction protein E-cadherin to distinguish the E-cadherin-positive basal stratum malpighii layer from the apical, non-viable, E-cadherin-negative stratum corneum layer. The stratum malpighii layer was similar between the groups. The pIUD group displayed a significantly thinner stratum corneum as compared to the noHC group, but not compared to the COC group.

To further explore the influence of progesterone-based HC use on the barrier function of the ectocervical epithelium, qPCR was used to assess mRNA levels of epithelial junction markers. While the mRNA levels for E-cadherin, claudin-1 and occludin were similarly expressed across the study groups, the level of ZO-1 mRNA was significantly lower in the pIUD users compared to the COC users, and trended lower as compared to the noHC users.

Immunohistochemistry and image analysis were further used to measure the presence of HIV target cells. CD4⁺, CCR5⁺, and Langerin⁺ cells were present in the ectocervical epithelium of all the individual tissue samples from the three study groups. A small number of DC-SIGN⁺ cells were detected just below the basal membrane, in agreement with previous studies in FGM showing that DC-SIGN⁺ cells are not located in the epithelium, but in the submucosal tissue compartment (43, 125). Furthermore, no differences were seen in the frequencies of the positively stained HIV target cells between the three study groups. Within the ectocervical epithelium the CD4⁺ cells were the most abundant cell followed by CCR5⁺ cells, Langerin⁺ cells whereas DC-SIGN⁺ cells showed close to nil abundance.

Collectively, these data show that women using pIUD displayed a thinner apical layer of the ectocervical epithelium and reduced ZO-1 expression as compared to the two control groups; the COC and the noHC group. At the same time, similar expression levels of HIV receptors and co-receptors were observed in the three study groups. These data suggest that pIUD use may weaken the ectocervical epithelial barrier against invading pathogens, including HIV. However, no multivariate analysis was performed, which would have been relevant in this study. Although down-regulation of a single protein may be compensated by higher expression of other proteins, this relative ZO-1 deficiency may affect the resistance of the mucosal barrier against HIV entrance, which may be deleterious from an HIV-transmission point of view.

Immune cells, including HIV target cells, may be differentially distributed under the influence of exogenous sex hormones but may also be affected by the endogenous sex hormones, therefore two control groups were included in this study. pIUD usage did not seem

to have a significant impact on HIV target cells in the mucosa, however there are recent studies showing that DMPA increase susceptibility to primary HIV infection as well as HIV shedding and transmission in already infected women (36, 93-95). Thus, future studies using larger study cohorts and experimental studies using cervical explant models to evaluate the effect of exogenous sex hormones including DMPA are needed.

Paper III. The first line of defense in the FGM against invading pathogens includes cervicovaginal fluids, commensal microbiota and a layer of mucus covering the cervical epithelium. Soluble factors including AMPs with microbicidal activity against a broad range of pathogens are present in genital secretions, as well as within mucosal tissue, and contribute to preventing infections such as HIV (59). Hence, in this study, we investigated how the use of different hormonal contraceptives affects the levels of AMPs in compartments of the FGM (tissue vs. cervical secretions). Healthy female volunteers using pIUD or COC from the Karolinska cohort were included. The study groups were similar in clinical characteristics including age, STIs and systemic medications.

The levels of the selected AMPs (HNP1-3, BD-2, LL-37, SLPI and trappin-2) were assessed in the tissue compartment (ectocervical biopsies) by qPCR and immunohistochemistry, and in the secretions (CVS) by ELISA. The qPCR analysis revealed that SLPI and trappin-2 were the most abundant AMPs in the ectocervical tissue, followed by BD-2, HNP1-3 and LL-37. Furthermore, women using COC had significantly lower mRNA levels of BD-2 and trappin-2 than pIUD users, while the mRNA levels of the other three AMPs were similar between the two study groups. The mRNA levels of HNP1-3 and LL-37 correlated significantly in both study groups.

Visual inspection of the immunohistochemical staining displayed that HNP1-3, SLPI and trappin-2 seemed to be more abundant than LL-37 and BD-2. However, no quantitative image analysis was performed due to lack of a standardized way to quantify staining intensity at that time. Positive staining for HNP1-3, BD-2, LL-37 and SLPI were seen both in the epithelium and in the submucosa while positive staining for trappin-2 were only seen in the epithelium. Furthermore, HNP1-3 and LL-37 showed overlapping staining patterns.

In the CVS samples HNP1-3 was the most abundant AMP, followed by SLPI, trappin-2, LL-37 and BD-2. Similar levels of the five AMPs were seen in the two study groups. Interestingly, a significant positive correlation between HNP1-3 and LL-37, as seen for the mRNA levels, were also seen, in both study groups, for the protein concentration in the CVS of these AMPs. Both HNP1-3 and LL-37 are important effector molecules secreted by neutrophils at mucosal sites (126) and our results may imply that their expression is coordinated in the FGM. However, if and how this is regulated by sex hormones remains to be further investigated

The reduction in the mRNA expression of BD-2 and trappin-2 in the ectocervical tissue seen in the COC users was not accompanied by a significant reduction at the protein level of BD-2 and trappin-2 in the corresponding CVS samples. The functional relevance of the difference in mRNA levels of BD-2 and trappin-2 between the groups needs to be further explored as well as the discrepancy between protein concentration in CVS of the AMPs and their mRNA expression in ectocervical tissue. This discrepancy may be due to post-translation- and secretion modifications such as proteolytic cleavage, or/and by the various sources of the CVS including the cervical vestibular glands, plasma transudate, and endometrial and oviductal fluids (127, 128).

Collectively, these results suggest that the impact of sex hormones on local immune defenses varies in different compartments (i.e. tissue vs. secretions) of the FGM. This finding shows the importance of examining tissue specimens in addition to genital secretions in order to adequately evaluate the effect of sex hormones on the local immune defenses of the FGM. The expression of these immunologic mediators within mucosal tissue at the site of infection may be as important as secretions in the immune defense of the host, but in a later stage. In this respect, the effect of hormonal contraception on susceptibility to infection may be more relevant in one compartment of the mucosa than in the other and may also vary across the female genital tract. Further studies including samples from multiple genital anatomical regions of the same individual is necessary to assess whether AMP expression is truly compartmentalized between mucosal tissue and secretions, as the result of a variable immunoregulatory effect of sex hormones in the female genital tract.

Paper IV. The female genital tract is a critical site for sexual transmission of HIV, and a number of genetic and immunological correlates of relative resistance against infection have been described (**Table 1**). However, the phenotype of relative resistance of HESN at the local genital site of sexual mucosal HIV infection is not fully understood. Hence, in this paper we investigated if epithelial thickness and quantity/localization of HIV target cells in ectocervical epithelium were linked with the relative resistance against HIV associated with the HESN phenotype in the Pumwani cohort comparing HESN to NN women.

The HESN women had been active in sex work for a median of 10 years and the NN women for a median of 2 years. The study groups were comparable for an extensive number of demographic parameters including STIs, however the HESN group were significantly older than the NN group and had experienced a higher number of pregnancies than the NN group.

In order to investigate if the ectocervical epithelial thickness differed between the study groups, immunofluorescence staining was performed. Staining for the adhesion junction protein E-Cadherin was used to visualize the ectocervical epithelium and its two major layers stratum; malpighii, corresponding to the epithelial layer expressing of E-Cadherin, and the

stratum corneum, corresponding to the apical layer associated with lack of E-Cadherin. Digital image analysis was used to measure the thickness of the total epithelium, as well as the thickness of the two different layers of the epithelium. No significant differences were seen between the study groups, in none of the three comparisons. The HIV receptors CD4, CCR5 and Langerin were next assessed in the epithelium in two study groups and the immunofluorescence staining revealed that CD4⁺, CCR5⁺ and Langerin⁺ cells were present in all the individual tissue samples representing the two study groups. Staining of CD4 together with CCR5 was performed for identification of the main HIV target cells, and CD4 was stained together with Langerin for identification of LCs in the ectocervical epithelium. The quantity of these receptors was assessed by computerized image analysis, and the results showed that there were no significant differences in expression of any of the cell markers between the study groups.

Next the spatial distribution of the CD4⁺, CCR5⁺, Langerin⁺ including CD4⁺CCR5⁺ and CD4⁺Langerin⁺ cells within the ectocervical epithelium were assessed by dividing the epithelium into 50µm segments. No statistical differences were seen, in none of the 50µm segment, between the study groups, for any of the cell markers assessed. To further investigate the spatial localization of HIV target cells, the distribution of CD4⁺CCR5⁺ and CD4⁺Langerin⁺ was compiled for the two study groups, and the two sets of staining showed that CD4⁺Langerin⁺ cells were localized more apical as compared with the CD4⁺CCR5⁺ cells.

We reasoned that a thick epithelium and low numbers of HIV target cells at a distant location far from the epithelial surface may contribute to relative resistance in the HESN phenotype. However, the results showed that this phenotype was not associated with an altered epithelial thickness, nor with altered levels or distributions of HIV target cells in the ectocervical epithelium.

The finding of an apical distribution of CD4⁺Langerin⁺ cells near the vaginal lumen was expected, since the main role for DCs is to seize incoming pathogens. However, it is unclear if an apical localization of LCs may enhance or reduce HIV susceptibility. Both skin-based and FGM-based studies showed that LCs can serve as primary target cells for HIV (129, 130), but also be a part of a protective barrier against HIV infection and transmission (47, 131, 132). However, Pena-Cruz *et al* recently showed that vaginal epithelial DCs did not harbor Birbeck granules and therefore are potentially both infected early during heterosexual transmission and retain virus during treatment (46). A lack of Birbeck granules may explain why HIV is commonly acquired across mucosal surfaces but not from exposed skin (46). Vaginal epithelial DCs may thus have unique characteristics as compared to skin-based LCs, which need to be further investigated.

In this study, the potential mucosal immune correlates of the relatively resistant HESN phenotype was explored in unique ectocervical tissue samples. The HESN group was compared with women who had been involved in sex work for three years or less in the same cohort. Nevertheless, we cannot exclude that some of the NN will become HESN and have the same relative resistant phenotype, and it would be interesting to conduct a longitudinal follow-up study on these women considering the frequency of HIV seroconversion. Thus, further studies are needed to reveal multifactorial and individual mechanisms of protection against infection linked to the HESN phenotype.

5 OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

A better understanding of how asymptomatic HSV-2 infection, progesterone-based HC and relative resistance to HIV (the HESN phenotype) affect the genital mucosa is important for development of novel prophylactic compounds with the ultimate goal of lowering the risk of global HIV transmission. In males, the foreskin mucosa is a highly relevant site for investigating viral transmission since circumcision reduces the risk of acquiring HIV infection (133). Here we have studied a representative population of young men living in an HIV endemic region in Kenya (114). In females, the cervix is an important site of sexual HIV transmission since it is directly exposed to the penile shaft and HIV-containing seminal fluid during vaginal intercourse. The FGM is influenced by many external factors that differs between geographical regions, such as STI patterns, and therefore we have included women from both Kenya and Sweden. Women who are at high risk of HIV exposure by engaging in sexual activities with HIV infected partners have been seen to have a different mucosal inflammatory status than low risk women (134).

The main purpose of this thesis was therefore to investigate how asymptomatic HSV-2 infection, progesterone-based HC, and long-term risk of sexual HIV exposures affect the genital epithelial barrier and the mucosal immune system in order to better understand HIV susceptibility factors. A close epidemiological association between HSV-2 and HIV has been consistently reported (87), however, there is more to learn about the underlying mechanisms of how HSV-2 infection enhances HIV acquisition. Our results show that HSV-2 infection may have an impact on the epithelial integrity of the foreskin, and therefore strengthening the genital epithelial barrier at the local site could be one way of reducing the risk of HIV infection.

Today, there is an ongoing debate how various progesterone-based hormonal contraceptives affect male-to-female HIV transmission. We here extended the knowledge by investigating this effect of the ectocervical region in *ex vivo* studies. Our findings suggested that progesterone-based hormonal contraceptive use may have an impact on the ectocervical epithelial thickness and integrity which could theoretically enhance HIV susceptibility. However, the most common contraceptive method in HIV-endemic Sub-Saharan Africa today is the injectable DMPA which contains higher levels of progesterone than the progesterone-based HC used in our studies (135). DMPA use has been associated with increased HIV susceptibility and HIV shedding in already infected women (95, 97, 136-138), and therefore it would be interesting to evaluate its impact in the ectocervical region. Further evaluation of the effects of contraceptive use on HIV susceptibility and studies on other FGM sites, other study cohorts and other progesterone-based HCs are essential and could guide WHO in establishment of adequate guidelines to prevent HIV transmission.

A number of immunological correlates with the HESN phenotype within the genital mucosa have been identified in cervical mononuclear cells and cervicovaginal lavage. However, in

this thesis we explored HIV susceptibility factors in unique tissue samples from HESN women showing that the HESN phenotype was neither associated with epithelial thickness nor with the localization/distribution of HIV target cells. There may be a complex multifactorial explanation of the HESN phenotype. Identifying further mucosal immune correlates of the HESN phenotype at other sites in the female genital tract including other structural and immune markers would be an important step against development towards new HIV prevention strategies.

There is a gap in knowledge in how the initial HIV transmission occur and what factors that influence HIV susceptibility at the local site of infection. Our studies hopefully have contributed to an increased understanding of the complex dynamics of HIV susceptibility at local genital sites. These studies have been possible due to access to unique samples from the FGM; the actual site of sexual exposure. Within the female genital tract, we showed variations in expression of immune markers in CVS and adjacent tissue illustrating the complexity even at the local site. This emphasizes the importance of examining tissue specimens in addition to genital secretions to adequately address the effect of factors influencing local immune defenses of the FGM. The present projects, and parallel activities in our international network, may hopefully lead to deeper insights into the local mucosal milieu which is of major importance in order to prevent sexual transmission of HIV. Based on the results, it might be possible to identify new ways of strengthening the epithelial barrier by topical compounds at the local site, and thereby limit sexual transmission of HIV.

There are also additional sociological and behavioral aspects strongly affecting the HIV epidemic. Therefore, all potential intervention strategies must be implemented by considering the affected populations including gender. In Sub-Saharan Africa three in four new HIV infections are among girls aged 15–19 years (18). Furthermore, young women in Sub-Saharan Africa aged 15–24 years are twice as likely to be living with HIV as men which can be associated with various social and cultural factors (18). Women are exposed to more violence in their relationships, which together with their lower socioeconomic status and discrimination cause power imbalance. In some relationships, women often cannot take their own decisions about safer sexual practices, including use of contraception (139). In this perspective, implementation of antiviral drug administration with topical gels may be valuable and may furthermore allow women in taking their own HIV prevention actions. Furthermore, this would be of great benefit for FSW, since the HIV prevalence in this key population is 13 times higher as compared to the general population (21). In conclusion, effective protection of women would have a great impact on the global HIV epidemic and thus should be considered a high priority

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Idag lever cirka 37 miljoner människor med humant immunbristvirus (HIV) varav de flesta lever i södra Afrika. De första fallen av AIDS beskrevs sommaren 1981 och sedan dess har över 35 miljoner människor dött till följd av infektionen. Trots 37 års forskning och miljarder spenderade dollar är HIV ett virus som fortfarande förbryllar och överlistar världens forskare. Det finns idag inget botemedel eller vaccin mot HIV, men sedan mitten av 1990-talet kan man bromsa/förhindra utvecklingen av AIDS med effektiva bromsmediciner. Trots detta har endast lite mer än hälften av alla som lever med HIV tillgång till bromsmediciner.

Den vanligaste smittvägen för HIV är oskyddat samlag och för kvinnor verkar livmoderhalstappen (ectocervix) vara mottaglig/utsatt för infektion. HIV kan även överföras från infekterad moder till barn under graviditet samt vid förlossning och amning, eller via annan blodsmitta. Undantaget oskyddat analsex, så är risken att få HIV vid oskyddat samlag endast cirka 1:200 vilket troligtvis beror på naturligt skyddande faktorer i slemhinnans immunförsvar. Vid oskyddat samlag utsätts vaginala, anala och/eller orala slemhinnorna för viruset, varför det är viktigt att forska kring vilka faktorer som kan bidra till ett eventuellt skydd mot HIV i dessa slemhinnor.

För att ta reda på dessa faktorer studeras grupper som är utsatta för HIV men som lyckas undgå att bli infekterade (HIV exposed seronegative: HESN), exempelvis prostituerade kvinnor i Afrika, för att man tror att framförallt HESN-gruppen har ett naturligt starkare försvar mot HIV än andra. Både ärftliga och icke-ärftliga faktorer studeras med förhoppningen om att förstå mer exakt hur deras immunförsvar skyddar mot HIV, för att sedan kunna använda denna kunskap i framställningen av ett framtida vaccin mot viruset.

Tidigare studier har visat att det finns faktorer som påverkar hur mottaglig man är för att smittas av HIV. Exempelvis har man sett ett samband mellan olika könssjukdomar som till exempel herpes typ 2 (HSV-2) och en ökad risk för att bli HIV-infekterad. Även det kvinnliga könshormonet progesteron, som ofta ingår i olika typer av preventivmedel, har visat sig öka risken för HIV-infektion i ap-modeller. Mekanismerna bakom den ökade risken är fortfarande inte kända och återstår att klarläggas.

Vid en HIV-infektion måste viruset ta sig igenom naturligt förekommande fysiska och immunologiska barriärer i vagina. Fysiska barriärer såsom den tjockflytande vätskan (sekretet) på slemhinnan och en intakt yttre del på slemhinnan (epitel). Epitelet hålls intakt med hjälp av proteiner såsom Claudin, Occludin, E-Cadherin och ZO-1 vilket försvårar för viruset att ta sig in. Sekretet innehåller dessutom olika lösliga molekyler såsom antimikrobiella peptider (AMPs) som verkar skyddande mot HIV. Om viruset, trots dessa skyddsbarriärer, lyckas ta sig igenom kan det binda och infektera dess specifika mål-celler CD4⁺T lymfocyter, dendritiska celler (DCs) och makrofager. För att en infektion av mål-cellen ska kunna ske, så måste viruset först binda till ett ytprotein, CD4, och sedan även ytterligare ett protein CCR5 eller CXCR4. Förutom via dessa proteiner, så kan även DCs och

makrofager binda HIV med andra yt-protein så kallade C-type lectin receptorerna (CLRs) Langerin, DC-SIGN och Mannose Receptor (MR). Om DC-SIGN binder till viruset så ökar risken att smittas eftersom CD4⁺T cellerna i sin tur infekteras av överfört virus.

Syftet med denna avhandling var att undersöka hur asymtomatisk HSV-2 infektion, progesteron-baserade preventivmedel och exponering för HIV påverkar barriärerna och immunsystemet i genitala slemhinnorna, med målet att bättre förstå vilka faktorer som påverkar mottagligheten för HIV-infektion.

Den första studien inkluderade förhudsbiopsier från asymtomatiska HSV-2 positiva och HSV-2 negativa män som genomgått omskärelse och resultaten visade att det inte förelåg en generell inflammation hos de HSV-2 positiva männen, dock uttryckte de lägre nivåer av Claudin-1 vilket skulle kunna tyda på en mindre robust barriär. Ett intakt epitel är nödvändigt för att skydda mot sexuellt överförbara infektioner såsom HIV, möjligtvis skulle lägre uttryck av Claudin-1 kunna vara en del av förklaringen till att HSV-2 är förknippat med ökad risk för HIV.

I den andra studien undersöktes om användningen av pIUD var associerat med ett tunnare epitel, lägre uttryck av proteiner som upprätthåller ett intakt epitel och fördelningen av målceller i ectocervixbiopsier från svenska kvinnor. Resultaten visade att pIUD-gruppen hade ett tunnare yttersta epitellager i ectocervix och lägre uttryck av ZO-1 jämfört med kontrollgrupperna, även om det inte var någon skillnad gällande uttryck av målceller. Detta skulle kunna tyda på att pIUD-användning kan leda till en försvagad barriär mot virus inklusive HIV i ectocervix.

I tredje studien undersöktes huruvida olika hormonella preventivmedel innehållandes progesteron påverkar produktionen av AMPs i både vaginala biopsier/sekret från svenska kvinnor. Resultaten visade att användandet av dessa preventivmedel påverkade produktionen av AMPs på olika sätt i vävnad jämfört med i sekret, vilket tyder på att progesteron påverkar lokala immunsvaret på olika sätt beroende på vävnad, sekret och olika lokalisation i vagina.

I fjärde studien undersöktes huruvida den skyddande HESN-fenotypen är associerad med minskning av målceller och tjockare skyddande epitel i ectocervixbiopsier från Kenyanska prostituerade kvinnor. Resultaten visade ingen skillnad mellan HESN-gruppen och kontrollgruppen gällande detta, dock befann sig Langerin-uttryckande celler yttligare i epitelet än CD4⁺CCR5⁺celler. Resultaten visade inga samband mellan HESN och förändrad epiteltjocklek/lokalisering av målceller i ectocervix, men fler studier behövs för att säkerställa vilka multifaktoriella, individuella faktorer som möjligen ligger till grund för den skyddande fenotypen.

Sammanfattningsvis, HSV-2 infektion och progesteron-baserade hormonella preventivmedel kan påverka genitala slemhinnan och eventuellt öka mottagligheten för HIV. Progesterons påverkan på lokala immunförsvaret är svårt att bedöma eftersom det varierar i olika delar av kvinnliga underlivets vävnad och sekret. Vidare har vi inte funnit stöd för att HESN-

fenotypen är kopplad till tjocklek av epitel eller av HIV-målcellernas lokalisation eller mängd, utan sannolikt är det många olika faktorer som bidrar till deras skydd mot HIV.

Denna avhandling, tillsammans med forskning i vårt internationella nätverk, kommer förhoppningsvis leda till en djupare förståelse för de lokala immunologiska mekanismerna i genital slemhinna som ger skydd mot HIV. Kanske kan vi i framtiden identifiera nya vägar för att stärka slemhinnebarriären genom att ta fram viktiga komponenter som skulle kunna appliceras lokalt i gel-form, och därmed minska sexuell HIV-smitta.

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